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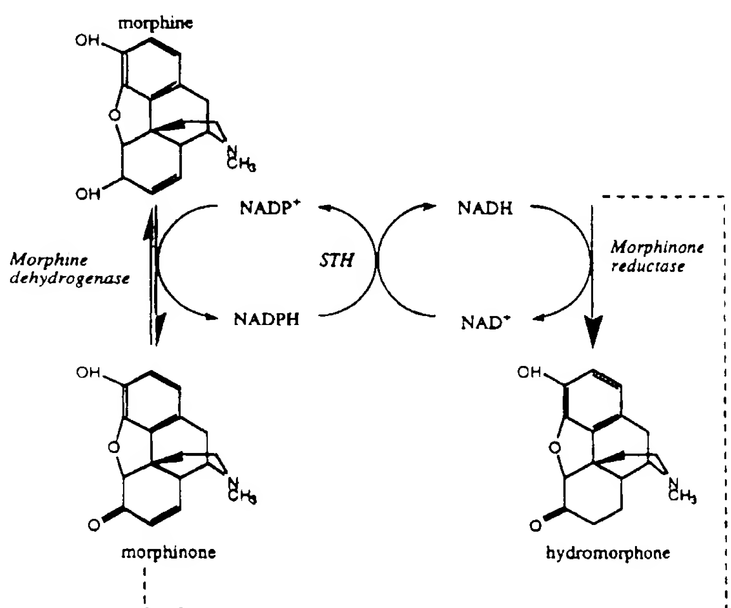
(54) Title: ENZYMATIC COFACTOR CYCLING USING SOLUBLE PYRIDINE NUCLEOTIDE TRANSHYDROGENASE

(57) Abstract

In an enzymic reaction involving a pyridine nucleotide cofactor, an enzyme is used that has sequence of greater than 70 % identity to SEQ ID No. 2 and capable of transferring reducing equivalents between pyridine nucleotide cofactors. Alternatively, a cell transformed to express the enzyme may be used.



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ENZYMATIC COFACTOR CYCLING USING SOLUBLE PYRIDINE NUCLEOTIDE TRANSHYDROGENASE

Field of the Invention

This invention relates to the use of an enzyme for the oxidation or reduction of pyridine nucleotide cofactors during enzymic reactions *in vivo* or *in vitro*, for example in enzymic or whole-cell biotransformations or enzymic analytical techniques.

Background of the Invention

Biotransformation procedures using natural or genetically-modified microorganisms or isolated enzymes provide methods for the synthesis of many useful products. Biotransformations have several advantages over chemical synthetic methods, in particular regiospecificity and stereospecificity of the enzyme-catalysed reactions, use of mild reaction conditions, and absence of requirement for toxic solvents.

Oxidoreductase enzymes often require redox-active cofactors for activity. Among the most common such cofactors are the pyridine nucleotide cofactors nicotinamide adenine dinucleotide (NAD: oxidized form NAD^+, reduced form NADH) and nicotinamide adenine dinucleotide phosphate (NADP: oxidized form $NADP^+$, reduced form NADPH). These cofactors are expensive and, except in the cases of extremely valuable products, cannot feasibly be supplied in stoichiometric quantities. This is one factor limiting the use of many oxidoreductase enzymes for biotransformation reactions.

The requirement for cofactors in a biotransformation process can be reduced by the provision of a means of regenerating the desired form of the cofactor. This means that the cofactor need be supplied only in catalytic quantities. For example, if the reaction of interest requires NAD^+, which is reduced in the reaction to NADH, the NADH can be re-oxidized by NAD^+ by another enzyme system, such as NAD^+-dependent formic dehydrogenase in the presence of formate. This is referred to as cofactor cycling. Formic dehydrogenase is particularly suitable for this purpose, since the reaction it catalyses is essentially irreversible.

A further complication is that the majority of NAD-requiring enzymes are not able to use NADP as a cofactor, and *vice versa*. For example, formic dehydrogenase could not be used to regenerate NADPH from $NADP^+$.

A special case is where a biotransformation process requires two oxidoreductase enzymes which require different cofactors. For example, a recently proposed biotransformation process for the conversion of morphine to the powerful painkiller hydromorphone requires the sequential action of $NADP^+$-dependent morphine dehydrogenase and NADH-dependent morphinone reductase (French *et al* (1995) Bio/Technology **13**:674-676). In the first reaction, morphine is converted to morphinone with reduction of $NADP^+$ to NADPH, and in the second reaction morphinone is converted to hydromorphone with oxidation of NADH to NAD^+. Therefore, both $NADP^+$ and NADH must be supplied. A further complication is that, in the presence of NADPH generated in the first reaction, morphine dehydrogenase reduces the product hydromorphone to an undesirable product, dihydromorphine, with re-oxidation of NADPH to $NADP^+$. These reactions are shown in the accompanying Figure 1A.

Pyridine nucleotide-dependent enzymes can also be used in certain enzymic assay procedures, with the quantity of the analyte being determined by the degree of oxidation or reduction of the cofactor. Oxidation and reduction of NAD and NADP can be measured by several methods; for example, spectrophotometry and fluorimetry. However, exceptionally sensitive methods for detecting oxidation or reduction may only be available for either NAD or NADP, but not both. For example, the oxidation of NADH to NAD^+ can be detected with extreme sensitivity by using the enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerokinase (PGK) to phosphorylate adenosine diphosphate (ADP) to adenosine triphosphate (ATP) in a reaction dependent on the presence of NAD^+, and then detecting the resulting ATP by the ATP-dependent light-emitting reaction of firefly luciferase. This method cannot be used to detect oxidation of NADPH to $NADP^+$, since the commercially available GAPDH is specific for NAD^+.

Several of the problems mentioned above can be overcome by the use of an enzyme which transfers reducing equivalents between NAD and NADP; for example, reducing NAD^+ to NADH while oxidizing NADPH to $NADP^+$. Such an enzyme is known as a pyridine nucleotide transhydrogenase (PNTH). Several types of enzyme exhibit this activity (Rydstrom *et al* (1987) in 'Pyridine nucleotide coenzymes: chemical, biochemical and medical aspects', part B, eds. Dolphin *et al*, John Wiley and Sons, NY, p.433-460). The best known is the membrane-bound, proton-pumping PNTH found in the membranes

of mitochondria and certain bacteria such as *Escherichia coli*. This enzyme, being membrane-bound, is generally unsuitable for biotransformation and analytical purposes. Soluble, non-energy-linked PNTH has been reported to occur in certain bacteria such as *Pseudomonas fluorescens*, *Pseudomonas aeruginosa* and *Azotobacter vinelandii*. This enzyme has been characterized in some detail, but its utility is limited.

Summary of the Invention

The gene (designated *sth*) encoding the soluble transhydrogenase of *Pseudomonas fluorescens* NCIMB 9815 has been cloned and sequenced, and the enzyme has been overexpressed in *Escherichia coli*. This enables the preparation of large amounts of enzyme relatively easily. The enzyme has been purified and characterized. This enzyme is defined by the reaction it catalyses, namely, transfer of reducing equivalents between NAD and NADP or analogues of these cofactors; the nucleotide sequence of the structural gene, *sth*, encoding the enzyme, and the deduced amino acid sequence of the enzyme derived therefrom; structural properties of the enzyme, including a subunit M_r of approximately 50,000; and the capacity to form large polymers of M_r exceeding 1,000,000.

According to a first aspect of this invention, the enzyme is used to act upon pyridine nucleotide cofactors so as to enhance a biotransformation process, for example, to alter the oxidation state of NAD or NADP or analogues of these cofactors. This may be so as to allow the action of another enzyme upon these cofactors. Alternatively, an altered form of the enzyme, prepared by random or site-directed mutagenesis of the structural gene, might be used. Such an altered enzyme may show altered levels of activity, altered regulation, or altered subunit structure.

The gene *sth* constitutes a second aspect of this invention. The gene may be used for the production of the enzyme or an altered form of the enzyme using a genetically modified organism. For example, a genetically modified organism carrying the *sth* gene as all or part of a heterologous construct may be grown in such a way as to encourage production of the enzyme, which may then be recovered from the culture medium or from cell extracts. The methods for accomplishing this are well known in the art.

A third aspect of this invention is the genetically modified organism which expresses the enzyme. Such an organism may be used in a whole cell biotransformation process

which may be enhanced by the presence in the cells of the active enzyme. Techniques for generating such recombinant organisms are well known in the art.

According to a fourth aspect of the invention, the enzyme is used in enzyme-based analytical assays so as to enhance these assays. For example, the enzyme may be used to, in effect, convert a signal measured as oxidation of NADPH to $NADP^+$ to a signal that can be measured based on oxidation of NADH to NAD^+. The altered signal may thereafter be detected by a more sensitive technique which was not formerly applicable.

Description of the Invention

The invention may be utilised by the enzyme having the sequence shown in SEQ ID No.2, or an amino-acid sequence having more than 70%, preferably at least 80%, and more preferably at least 90% identity. The enzyme may be used as such, or as a transformed organism. Suitable hosts for transformation are well known to those of ordinary skill in the art. An example of a suitable host is *E. coli*.

An enzyme or organism of the invention may be used in biotransformation, for analytical purposes, or for any other appropriate purpose. It is particularly useful in connection with a reaction in which an enzyme uses a pyridine nucleotide cofactor. A specific example is shown in Fig. 1B (to be compared with Fig. 1A). The use of STH means that reduction of hydromorphone is greatly decreased, by avoiding a build-up of NADH. This eliminates the need to supply expensive cofactors. In biotransformation, STH may shuttle reducing equivalents from NADH to NAD^+, allowing cells to be used in the process more than once.

The following Example 1 illustrates the cloning and sequencing of *sth*, while Examples 2 and 3 illustrate the use of STH in accordance with the invention. The Examples are given with reference to Fig. 1 (described above) and the other accompanying drawings, in which:

Figure 2 is a restriction map of the 5.0 kb *Eco* RI fragment and the 1.5 kb *Sac* II/*Xho* I subclone bearing the *sth* gene. The shaded area indicates the coding region and arrows indicate sequencing reactions.

Figure 3 shows the transformation of morphine to hydromorphone in the presence of soluble transhydrogenase. Squares, morphine, circles, hydromorphone, triangles, dihydromorphine.

Figure 4 shows the consecutive morphine biotransformations with cells of *E. coli* JM109/pMORB3-AmutMC80S/pPNT4 and *E. coli* JM109/pMORB3-AmutMC80S (OC = Opiate Concentration (mM), □ = morphine, ● = hydromorphone and ▲ = dihydromorphine).

Example 1

Thionicotinamide adenine dinucleotide (tNAD+) and adenosine-2',5'-diphosphate agarose were obtained from Sigma (Poole, Dorset, UK). Other reagents were of analytical or higher grade and were obtained from Sigma or Aldrich (Gillingham, Dorset, UK).

Pseudomonas fluorescens NCIMB9815 was obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland, UK). *Escherichia coli* JM109 was obtained from Promega (Southampton, UK). Both organisms were routinely grown in SOB medium (Sambrook *et al* (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbour, NY) at 30°C (*P. fluorescens*) or 37°C (*E. coli*) with rotary shaking at 180 rpm.

STH activity was routinely assayed by observing the reduction of thionicotinamide adenine dinucleotide ($tNAD^+$), an analogue of NAD^+ with altered spectral characteristics, at 400 nm in a reaction mixture consisting of 0.1 mM $tNAD^+$ and 0.1 mM NADPH in 50 mM phosphate buffer, pH 7.0, at 30°C. One unit (U) of enzyme activity was defined as that amount of activity reducing 1 mmol of $tNAD^+$ per min under these conditions. The molar change in absorbance at 400 nm of $tNAD^+$ on reduction to tNADH was taken as 11 300 l $mol^{-1}cm^{-1}$ (Cohen *et al* (1970) J. Biol. Chem. **245**:2825-2836). Protein concentration was routinely assayed using the reagent of Pierce (Rockford, IL, USA) according to the manufacturer's protocol. Bovine serum albumin was used as a standard. Specific activity was calculated as units of STH activity per mg of protein (U/mg).

pBluescript SK+, a standard cloning vector, was obtained from Stratagene (Cambridge, Cambs., UK). pS 1EMBL, a low-copy number vector, is described in Poustka *et al* (1984) Proc. Natl. Acad. Sci. USA **81**:4129-4133. Southern blotting and DNA manipulation were performed using standard techniques (Sambrook *et al*, *supra*).

Purification of STH: Soluble pyridine nucleotide transhydrogenase (STH) was purified from cells of *P. fluorescens* NCIMB9815 according to a modification of the method of

Hojeberg *et al* (1976) Eur J Biochem 66 467-475. Cells were grown to stationary phase in 1 l of SOB medium. The cells were harvested by centrifugation (5000 g, 15 min) and resuspended in 20 ml buffer A (50 mM Tris/HCl, pH 7.0, with 2 mM dithiothreitol). The cells were then disrupted by sonication (25 bursts of 5 s at 12 μm separated by 30 s pauses for cooling in an ice-water bath) using an MSE Soniprep 150. Cell debris was removed by centrifugation (25,000 g, 10 min). The extract contained 93 units of STH activity at a specific activity of 0.19 U/mg.

STH was purified using a column of 1 cm inner diameter packed with 6 ml of adenosine-2',5'-diphosphate agarose (packed height 7.6 cm). The column was operated at 12 ml/h during loading and 24 ml/h during washing. All procedures were performed at 4°C and all buffers contained 2 mM dithiothreitol. After equilibration of the column with 5 mM $CaCl_2$ in buffer A, crude extract (20 ml), to which $CaCl_2$ had been added to a final concentration of 5 mM, was loaded onto the column. The column was then washed with 90 ml of 0.4 M NaCl, 5 mM $CaCl_2$ in buffer A, followed by 24 ml of 0.7 M NaCl, 5 mM $CaCl_2$ in buffer A. Bound *vice versa* was eluted with 50 mM tris/HCl, pH 8.9, containing 0.4 M NaCl. Fractions of 5 ml were collected and the active fractions were pooled. The pooled product was concentrated by ultrafiltration using an Amicon 8050 ultrafiltration cell fitted with a membrane of nominal M_r cutoff 10,000, and then diafiltered with buffer A to reduce the pH and salt concentration. The final volume was 1.5 ml. This material contained 62 U of STH activity at a specific activity of 140 U/mg.

This product was then applied to a gel filtration column of 1.6 cm inner diameter packed with 150 ml of Sephacryl S-300 (Pharmacia) (packed height 75 cm) equilibrated with buffer A. The column was operated at 8 ml/h. Fractions of 2 ml were collected. Active fractions (16 ml) were pooled and concentrated by ultrafiltration as described above to a final volume of 1 ml. The product contained 26 U of STH activity at a specific activity of 310 U/mg.

Prior to analysis by SDS-PAGE the sample was further concentrated by freeze-drying and resuspension in a small volume of buffer A. The reconstituted material was not active. SDS-PAGE showed a single protein band with an apparent M_r of 55,000, consistent with the value reported for the enzyme from *Pseudomonas aeruginosa* (Rydstrom *et al*, *supra*).

Cloning: Protein was transferred from an SDS-PAGE gel to poly(vinylidene difluoride) (PVDF) membrane (ProBlott, Applied Biosystems, Foster City, CA, USA) using the PhastTransfer semi-dry transfer system (Pharmacia, St. Albans, Herts., UK) according to the manufacturer's instructions

The N-terminal sequence was determined by automated Edman degradation. The N-terminal sequence of the purified PNTH was determined as:

A-V-Y-N-Y-D-V-V-V-L-G-S-(G/V)-P-A-G-E-(G/V)-A-A-M-N-A-A-(R/D)-

where parentheses indicate uncertain assignments

A codon bias table for *P. fluorescens* was derived based on 20 genes in the Gen-EMBL database. This revealed a significant preference for G and C in the third position for most codons. Based on this codon bias, the following degenerate oligonucleotide was designed: AC-(C/G)AC-(C/G)AC-GTC-GTA-GTT-GTA-(C/G)AC-(G/C)GC (based on residues 1 to 9 of the N-terminal sequence).

Southern blots of genomic DNA from *P. fluorescens* NCIMB9815 showed that this oligonucleotide bound most strongly to a 5.0 kb *Eco* RI fragment. A library of *Eco* RI fragments of 4 to 6 kb was prepared in the cloning vector pBluescript SK+ using *E. coli* JM109 as a host, and recombinant cells were screened by colony blotting using the oligonucleotide probe. Several positive colonies were isolated and all were found to bear the same 5.0 kb insert. Both orientations of the insert were recovered. The recombinant plasmids were designated pSTH1A and pSTH1B, varying only in the orientation of the *Eco* RI insert. The gene *sth* was localized by restriction mapping of the insert followed by Southern analysis using the oligonucleotide probe. Sequencing indicated the presence of an open reading frame encoding a protein of the same N-terminal sequence as that determined for STH. Various subclones were prepared in pBluescript SK+ and sequenced using vector-based primers as shown in Figure 1. The sequence of *sth* and the deduced amino acid sequence of STH are shown as SEQ ID Nos. 1 and 2.

Cell extracts prepared from saturated cultures of *E. coli* JM109/pSTH1A or pSTH1B showed detectable STH activity, assayed by the reduction of thionicotinamide adenine dinucleotide (tNAD^{+}) in the presence of NADPH. A 1.5 kb *Sac* II/*Xho* I fragment from pSTH1A was subcloned in pBluescript SK+ (Figure 2). This plasmid was designated pSTH2. In pSTH2, *sth* is in the correct orientation to be expressed from the *lac* promoter

of pBluescript SK−. Cell extracts from saturated cultures of *E. coli* JM109/pSTH2 in the absence or presence of 0.4 mM IPTG showed transhydrogenase activity of 4.1 U/mg and 22.0 U/mg respectively. Based on the specific activity of purified STH, it was estimated that in the latter case STH formed approximately 6% of soluble cell protein, approximately 100 times the level seen in *P. fluorescens*.

The recombinant STH was purified to apparent homogeneity in a single affinity chromatography step using adenosine-2',5'-diphosphate agarose. Cell extract was prepared as described above from 1 l of saturated culture of *E. coli* JM109/pSTH2 grown in the presence of 0.4 mM IPTG. Of the resulting 25 ml of cell extract, 5 ml, containing 2140 U of STH activity at a specific activity of 27 U/mg, was loaded onto a column packed with adenosine-2',5'-diphosphate agarose as described above. The column was washed with 35 ml of 0.7 M NaCl, 5 mM $CaCl_2$ in buffer A. STH was then eluted with 0.4 M NaCl in 50 mM Tris/HCl, pH 8.9. The most active fractions, totalling 13 ml, were pooled, concentrated and diafiltered as described above, except that a membrane of nominal molecular weight cutoff 300,000 was used. The product contained 900 U of STH activity at a specific activity of 300 U/mg. This material appeared to be homogeneous by SDS-PAGE; the gel-filtration step was therefore omitted. The purified STH was stored at -20°C in buffer A with 2 mM dithiothreitol, with no detectable loss of activity over several weeks.

The properties of the recombinant STH were compared to those reported for the enzyme from *Pseudomonas aeruginosa*. The subunit M_r as determined by SDS-PAGE is consistent with that previously reported (Rydström *et al*, *supra*). To determine whether the recombinant enzyme was capable of forming large polymers, samples were adsorbed to carbon films, negatively stained with 1% w/v uranyl acetate and examined by electron microscopy using a Phillips CM100 electron microscope. Long polymers of approximately 10 nm diameter and in excess of 500 nm long were observed. This is consistent with previous reports (Louie *et al* (1972) J. Mol. Biol. 70:651-664).

Example 2

Morphine dehydrogenase and morphinone reductase were prepared from recombinant strains of *Escherichia coli* according to published procedures (Willey *et al* (1993) Biochem. J. **290**:539-544; French and Bruce (1995) Biochem. J. **312**:671-678).

STH was prepared from *Pseudomonas fluorescens* NCIMB9815 as described in Example 1. Morphine alkaloids were quantified by HPLC (French *et al*, *supra*).

A reaction mixture consisting of 0.5 ml 50 mM Tris/HCl buffer, pH 8.0, containing 10 mM morphine, 0.2 mM NADPH, 0.2 mM NAD^+, 1 mM dithiothreitol, 1 unit of morphinone reductase, 1 unit of morphine dehydrogenase and 6 units of STH was incubated at 4°C for 8 hours. Samples of 50 µl were taken at intervals, treated with acetic acid to precipitate proteins, and analysed by HPLC. Morphine was converted to hydromorphone in high yield, as shown in Figure 3. A parallel experiment lacking STH was also performed. In this case, no transformation of morphine occurred. This demonstrates that STH is capable of catalysing cycling of cofactors in an enzymic biotransformation process.

Example 3

A 1.2 kb *Pst* I fragment bearing a mutant morphine dehydrogenase structural gene (*morA*) complete with its upstream ribosome binding site and promoter sequences was ligated into the low-copy number vector, pS 1EMBL, previously digested with *Pst* I creating the construct pMORA4mutMC80S, which contained suitable restriction sites for further subcloning. A 1.2 kb *Hin*dIII/*Eco* RI fragment carrying the mutant *morA* gene, ribosome binding site and promoter region was excised from pMORA4mutMC80S and ligated into *Hin*dIII/*Eco* RI-digested pMORB3 (French *et al*, *supra*) which carried a single copy of *morB*, the structural gene for morphinone reductase, together with its ribosome binding site and promoter region, creating the construct pMORB3-AmutMC80S.

A 1.5 kb *Pst* I/*Xho* I fragment bearing the structural gene for the soluble pyridine nucleotide transhydrogenase was ligated into pS 1EMBL, previously digested with *Pst* I and *Sal* I, creating the construct pPNT4.

Cells of *E. coli* JM109/pMORB3-AmutMC80S and *E. coli* JM109/pMORB3-AmutMC80S/pPNT4 were grown to stationary phase and harvested by centrifugation at 17,310 × g for 15 min at 4 °C. Cells were then washed with 50 mM Tris-HCl (pH 7.5) and recentrifuged. The supernatant was removed and the pelleted cells stored on ice until required for biotransformation. Typical values for enzyme activities in cells of *E. coli* JM109/pMORB3-AmutMC80S were 0.06 U/mg for morphine dehydrogenase and 0.88 U/mg morphinone reductase, whilst values in cells of *E. coli* JM109/pMORB3-

AmutMC80S/pPNT4 were 0.044 U/mg for morphine dehydrogenase, 0.78 U/mg for morphinone reductase and 0.72 U/mg for STH. Small scale whole cell biotransformations (3 ml total volume) were carried out in reaction mixtures containing 20 mM morphine and a final cell density of 0.17 g/ml in 50 mM Tris-HCl (pH 7.5). Biotransformations were carried out in duplicate at 30°C on a rotary shaker and samples taken at regular intervals. Samples were clarified by centrifugation and analysed for opiate content using HPLC as described previously (French *et al*, *supra*). A series of consecutive biotransformations were carried out using the same batch of cells which was harvested and washed between incubations. Results illustrated in Figure 4 indicate that cells containing recombinant STH were capable of being used more than once for the biotransformation process, while cells lacking recombinant STH could only be used once. These results imply that recombinant STH is capable of cofactor cycling in *in vivo* enzymic processes dependant on NADP and NAD.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(F) POSTAL CODE (ZIP): CB2 1TS

(ii) TITLE OF INVENTION: ENZYMIC COFACTOR CYCLING USING SOLUBLE PYRIDINE NUCLEOTIDE TRANSHYDROGENASE

(iii) NUMBER OF SEQUENCES: 2

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER: WO (not yet known)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1660 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS

(B) LOCATION:209..1[illegible]

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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                125                 130                 135

GTG GCC AAG CAC ATC ATC ATT GCC ACC GGC TCG CGC CCG TAT CGC CCG     664
Val Ala Lys His Ile Ile Ile Ala Thr Gly Ser Arg Pro Tyr Arg Pro
            140                 145                 150

GCG GAT ATC GAT TTC CAC CAC CCA CGT ATC TAC GAT AGC GAT ACC ATC     712
Ala Asp Ile Asp Phe His His Pro Arg Ile Tyr Asp Ser Asp Thr Ile
        155                 160                 165

CTC AGC CTG GGC CAC ACC CCA CGC AAA CTG ATC ATC TAT GGC GCC GGC     760
Leu Ser Leu Gly His Thr Pro Arg Lys Leu Ile Ile Tyr Gly Ala Gly
    170                 175                 180

GTC ATT GGC TGT GAA TAC GCC TCG ATC TTC AGC GGC CTG GGT GTG CTG     808
Val Ile Gly Cys Glu Tyr Ala Ser Ile Phe Ser Gly Leu Gly Val Leu
185                 190                 195                 200

GTC GAG CTG GTC GAC AAC CGC GAC CAG TTG CTG AGC TTC CTC GAC TCG     856
Val Glu Leu Val Asp Asn Arg Asp Gln Leu Leu Ser Phe Leu Asp Ser
                205                 210                 215

GAA ATC TCC CAG GCG TTG AGC TAC CAC TTC AGC AAC AAC AAC ATC ACT     904
Glu Ile Ser Gln Ala Leu Ser Tyr His Phe Ser Asn Asn Asn Ile Thr
            220                 225                 230

GTG CGC CAT AAC GAA GAG TAC GAT CGG GTC GAA GGC CTG GAC AAC GGG     952
Val Arg His Asn Glu Glu Tyr Asp Arg Val Glu Gly Leu Asp Asn Gly
        235                 240                 245

GTG ATC CTG CAC CTC AAG TCC GGC AAG AAG ATC AAG GCC GAC GCC TTG    1000
Val Ile Leu His Leu Lys Ser Gly Lys Lys Ile Lys Ala Asp Ala Leu
    250                 255                 260

CTG TGG TGC AAC GGT CGT ACC GGC AAC ACC GAC AAG CTG GGC ATG GAA    1048
Leu Trp Cys Asn Gly Arg Thr Gly Asn Thr Asp Lys Leu Gly Met Glu
265                 270                 275                 280

AAC ATC GGG GTC AAG GTC AAC AGC CGT GGC CAG ATC GAG GTG GAC GAA    1096
Asn Ile Gly Val Lys Val Asn Ser Arg Gly Gln Ile Glu Val Asp Glu
                285                 290                 295
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AAC TAC CGC ACC TGT GTG ACC AAC ATC TAT GGC GCC GGT GAC GTG ATC 1144
Asn Tyr Arg Thr Cys Val Thr Asn Ile Tyr Gly Ala Gly Asp Val Ile
300 305 310

GGC TGG CCG AGC CTG GCC AGT GCC GCC CAT GAC CAG GGC CGT TCG GCC 1192
Gly Trp Pro Ser Leu Ala Ser Ala Ala His Asp Gln Gly Arg Ser Ala
315 320 325

GCT GGC AGC ATC GTC GAC AAC GGC AGC TGG CGC TAT GTG AAC GAC GTA 1240
Ala Gly Ser Ile Val Asp Asn Gly Ser Trp Arg Tyr Val Asn Asp Val
330 335 340

CCG ACC GGG ATC TAC ACG ATT CCG GAG ATC AGC TCG ATC GGC AAG AAC 1288
Pro Thr Gly Ile Tyr Thr Ile Pro Glu Ile Ser Ser Ile Gly Lys Asn
345 350 355 360

GAA CAC GAA CTG ACC AAG GCC AAG GTG CCT TAC GAA GTG GGC AAG GCG 1336
Glu His Glu Leu Thr Lys Ala Lys Val Pro Tyr Glu Val Gly Lys Ala
365 370 375

TTC TTC AAG AGC ATG GCG CGT GCG CAG ATC GCC GGT GAG CCG CAA GGC 1384
Phe Phe Lys Ser Met Ala Arg Ala Gln Ile Ala Gly Glu Pro Gln Gly
380 385 390

ATG CTG AAG ATC CTG TTT CAC CGC GAG ACC CTG GAA GTC CTC GGC GTG 1432
Met Leu Lys Ile Leu Phe His Arg Glu Thr Leu Glu Val Leu Gly Val
395 400 405

CAT TGC TTC GGC TAC CAG GCT TCG GAG ATC GTG CAC ATC GGC CAG GCC 1480
His Cys Phe Gly Tyr Gln Ala Ser Glu Ile Val His Ile Gly Gln Ala
410 415 420

ATC ATG AAC CAG CCG GGC GAG CAA AAT ACC CTC AAG TAT TTC GTC AAC 1528
Ile Met Asn Gln Pro Gly Glu Gln Asn Thr Leu Lys Tyr Phe Val Asn
425 430 435 440

ACC ACC TTC AAC TAC CCG ACC ATG GCC GAA GCC TAT CGG GTA GCG GCC 1576
Thr Thr Phe Asn Tyr Pro Thr Met Ala Glu Ala Tyr Arg Val Ala Ala
445 450 455

TAC GAT GGC CTC AAC CGG CTT TTT TGAGCGGCTC CGGCCGGTGG CCTGAGCCGG 1630
Tyr Asp Gly Leu Asn Arg Leu Phe
460

CCGGGGAGAC CGATTTCAGT AATTCTCGAG 1660

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 464 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Ala Val Tyr Asn Tyr Asp Val Val Val Leu Gly Ser Gly Pro Ala
 1               5                  10                  15

Gly Glu Gly Ala Ala Met Asn Ala Ala Lys Ala Gly Arg Lys Val Ala
            20                  25                  30

Met Val Asp Ser Arg Arg Gln Val Gly Gly Asn Cys Thr His Leu Gly
        35                  40                  45

Thr Ile Pro Ser Lys Ala Leu Arg His Ser Val Arg Gln Ile Met Gln
    50                  55                  60

Phe Asn Thr Asn Pro Met Phe Arg Ala Ile Gly Glu Pro Arg Trp Phe
65                  70                  75                  80

Ser Phe Pro Asp Val Leu Lys Ser Ala Glu Lys Val Ile Ser Lys Gln
                85                  90                  95

Val Ala Ser Arg Thr Gly Tyr Tyr Ala Arg Asn Arg Val Asp Leu Phe
            100                 105                 110

Phe Gly Thr Gly Ser Phe Ala Asp Glu Gln Thr Val Glu Val Val Cys
        115                 120                 125

Ala Asn Gly Val Val Glu Lys Leu Val Ala Lys His Ile Ile Ile Ala
    130                 135                 140

Thr Gly Ser Arg Pro Tyr Arg Pro Ala Asp Ile Asp Phe His His Pro
145                 150                 155                 160
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```
Arg Ile Tyr Asp Ser Asp Thr Ile Leu Ser Leu Gly His Thr Pro Arg
                165                 170                 175

Lys Leu Ile Ile Tyr Gly Ala Gly Val Ile Gly Cys Glu Tyr Ala Ser
            180                 185                 190

Ile Phe Ser Gly Leu Gly Val Leu Val Glu Leu Val Asp Asn Arg Asp
        195                 200                 205

Gln Leu Leu Ser Phe Leu Asp Ser Glu Ile Ser Gln Ala Leu Ser Tyr
    210                 215                 220

His Phe Ser Asn Asn Asn Ile Thr Val Arg His Asn Glu Glu Tyr Asp
225                 230                 235                 240

Arg Val Glu Gly Leu Asp Asn Gly Val Ile Leu His Leu Lys Ser Gly
                245                 250                 255

Lys Lys Ile Lys Ala Asp Ala Leu Leu Trp Cys Asn Gly Arg Thr Gly
            260                 265                 270

Asn Thr Asp Lys Leu Gly Met Glu Asn Ile Gly Val Lys Val Asn Ser
        275                 280                 285

Arg Gly Gln Ile Glu Val Asp Glu Asn Tyr Arg Thr Cys Val Thr Asn
    290                 295                 300

Ile Tyr Gly Ala Gly Asp Val Ile Gly Trp Pro Ser Leu Ala Ser Ala
305                 310                 315                 320

Ala His Asp Gln Gly Arg Ser Ala Ala Gly Ser Ile Val Asp Asn Gly
                325                 330                 335

Ser Trp Arg Tyr Val Asn Asp Val Pro Thr Gly Ile Tyr Thr Ile Pro
            340                 345                 350

Glu Ile Ser Ser Ile Gly Lys Asn Glu His Glu Leu Thr Lys Ala Lys
        355                 360                 365

Val Pro Tyr Glu Val Gly Lys Ala Phe Phe Lys Ser Met Ala Arg Ala
    370                 375                 380

Gln Ile Ala Gly Glu Pro Gln Gly Met Leu Lys Ile Leu Phe His Arg
385                 390                 395                 400
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Glu Thr Leu Glu Val Leu Gly Val His Cys Phe Gly Tyr Gln Ala Ser
405 410 415

Glu Ile Val His Ile Gly Gln Ala Ile Met Asn Gln Pro Gly Glu Gln
420 425 430

Asn Thr Leu Lys Tyr Phe Val Asn Thr Thr Phe Asn Tyr Pro Thr Met
435 440 445

Ala Glu Ala Tyr Arg Val Ala Ala Tyr Asp Gly Leu Asn Arg Leu Phe
450 455 460

CLAIMS

1. An organism transformed to express an enzyme having a sequence of greater than 70% identity to SEQ ID No. 2 and capable of transferring reducing equivalents between pyridine nucleotide cofactors

2. An organism according to claim 1, wherein the enzyme is soluble pyridine nucleotide transhydrogenase.

3. Use of an organism according to claim 1 or claim 2, as a biocatalyst.

4. A nucleotide molecule having a sequence of greater than 70% identity to SEQ ID No.1, encoding an enzyme having the activity of soluble pyridine nucleotide transhydrogenase.

5. A process in which a substrate is converted to a product by means of an enzyme and a pyridine nucleotide cofactor, which comprises the use of an enzyme or organism as defined in claim 1 or claim 2.

6. A process according to claim 5, which is a biotransformation or assay.

7. A process according to claim 5 or claim 6, wherein the substrate is morphine.

8. A process according to any of claims 5 to 7, which the cofactor is used in a catalytic amount.

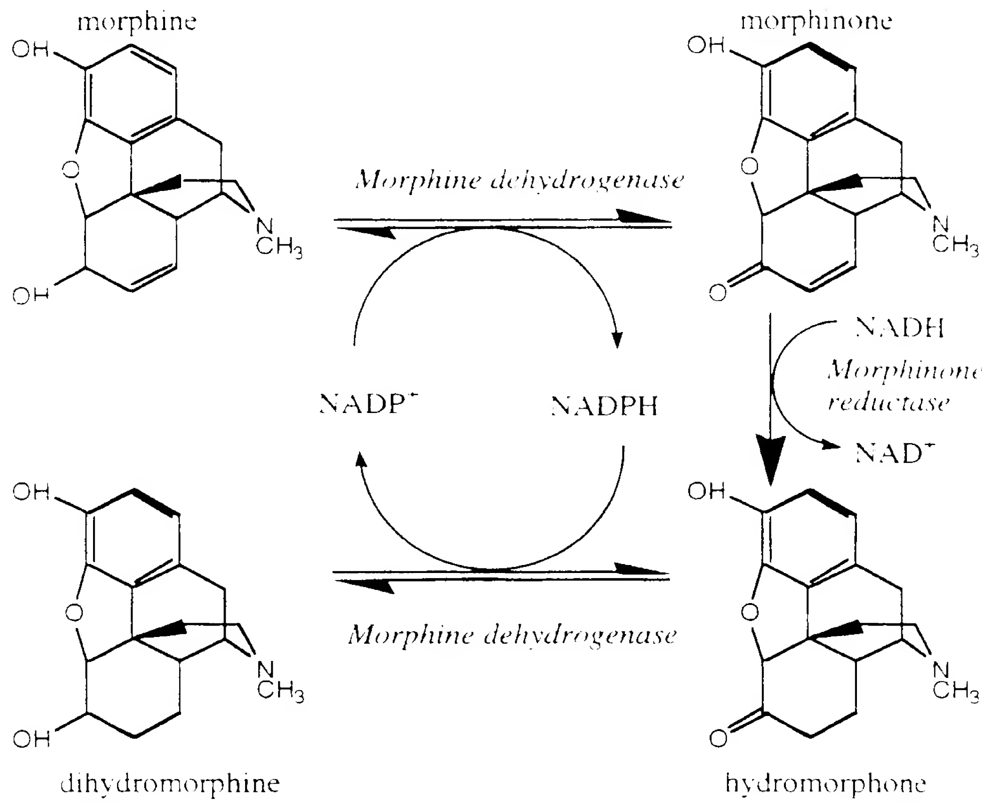
morphine
OH
O
N
CH3
OH
morphinone
OH
O
N
CH3
O
Morphine dehydrogenase
NADP+
NADPH
NADH
Morphinone
reductase
NAD+
OH
O
N
CH3
OH
dihydromorphine
OH
O
N
CH3
O
hydromorphone
Morphine dehydrogenase

FIGURE 1A

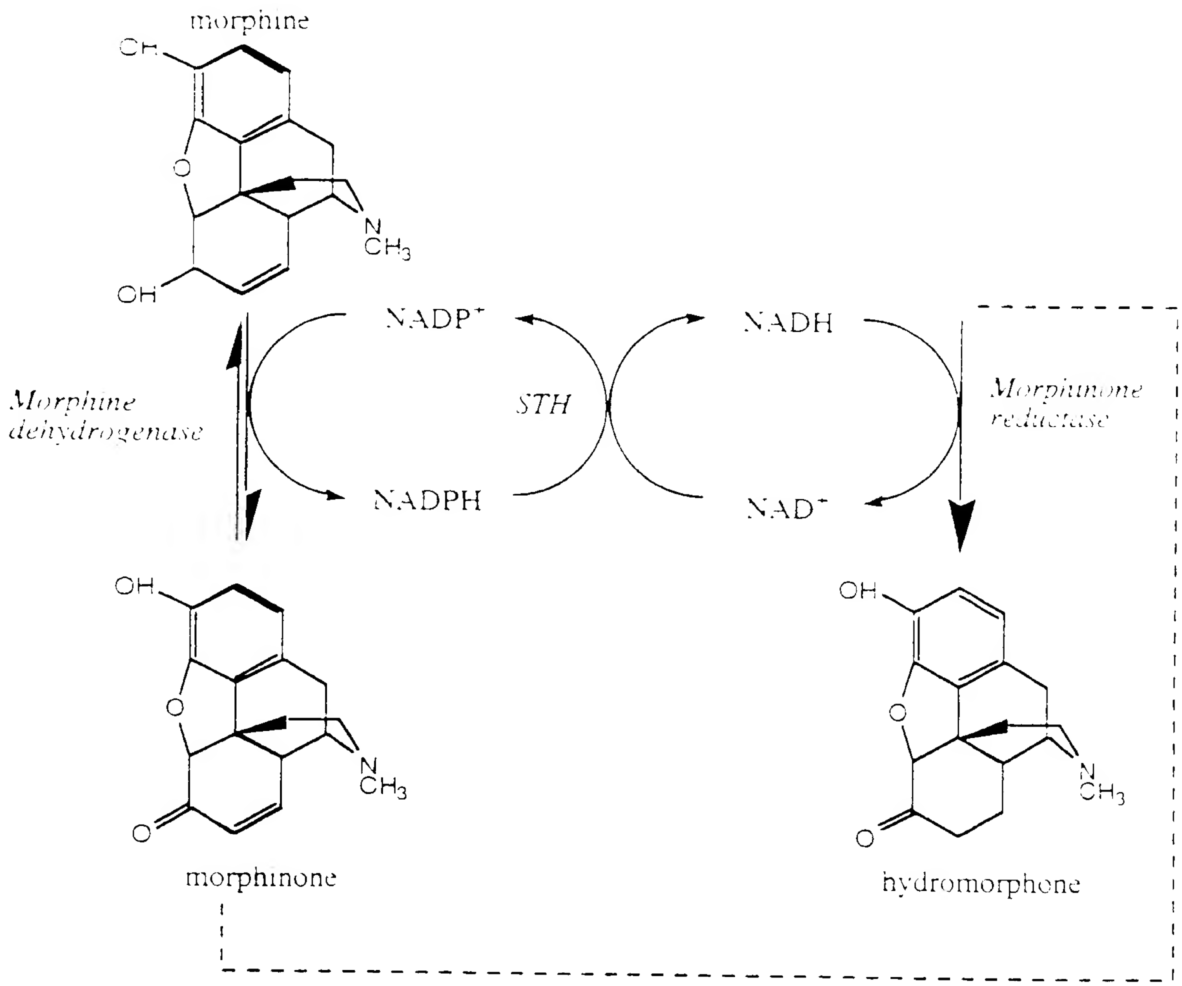
morphine
OH
O
N
CH3
OH
NADP+
NADH
Morphine
dehydrogenase
STH
Morphinone
reductase
NADPH
NAD+
OH
O
N
CH3
O
morphinone
OH
O
N
CH3
O
hydromorphone

FIGURE 1B

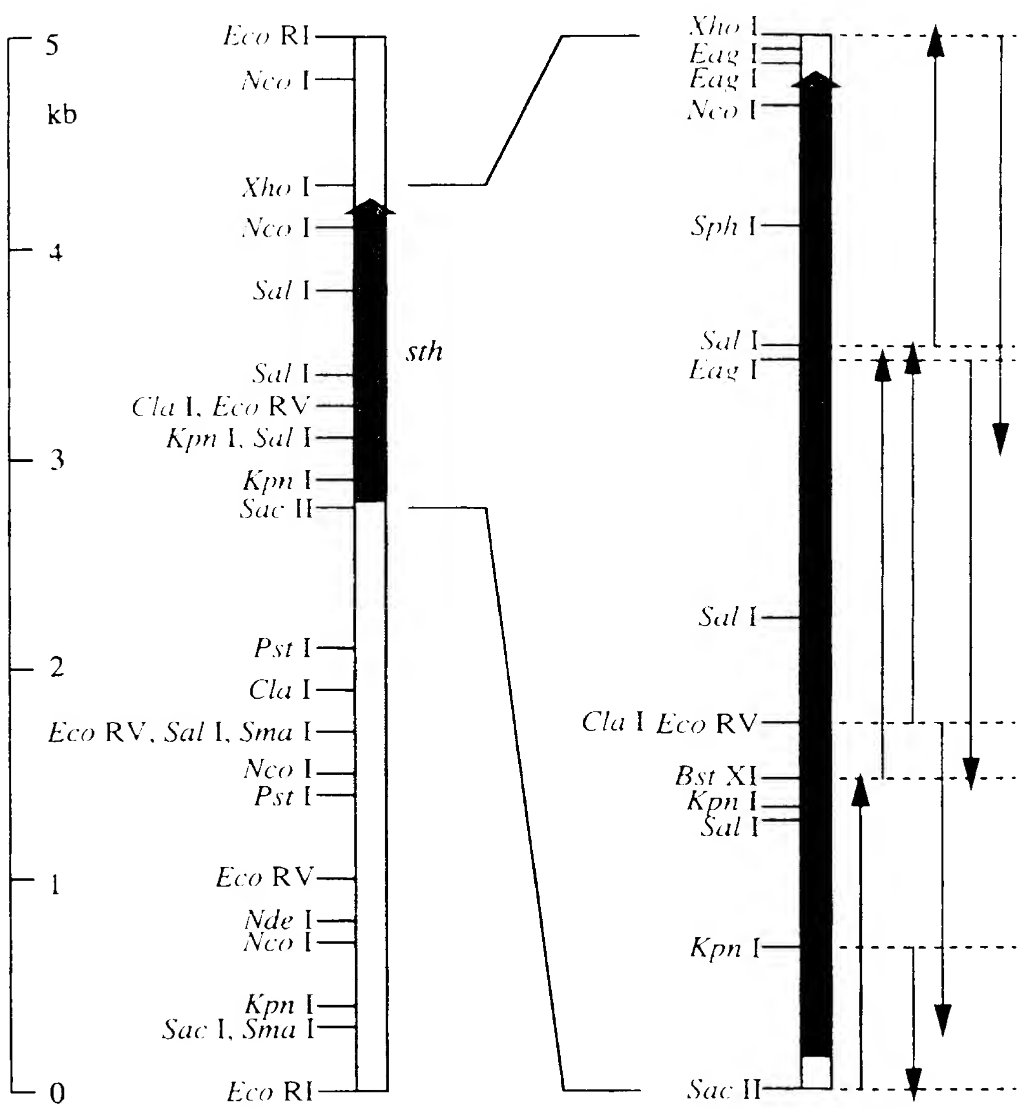
5
kb
4
3
2
1
0
Eco RI
Nco I
Xho I
Nco I
Sal I
Sal I
Cla I, Eco RV
Kpn I, Sal I
Kpn I
Sac II
Pst I
Cla I
Eco RV, Sal I, Sma I
Nco I
Pst I
Eco RV
Nde I
Nco I
Kpn I
Sac I, Sma I
Eco RI
sth
Xho I
Eag I
Eag I
Nco I
Sph I
Sal I
Eag I
Sal I
Cla I Eco RV
Bst XI
Kpn I
Sal I
Kpn I
Sac II

FIGURE 2

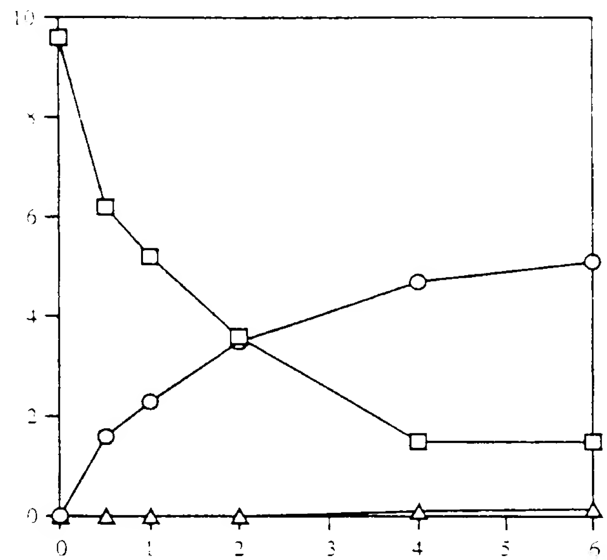

FIGURE 3

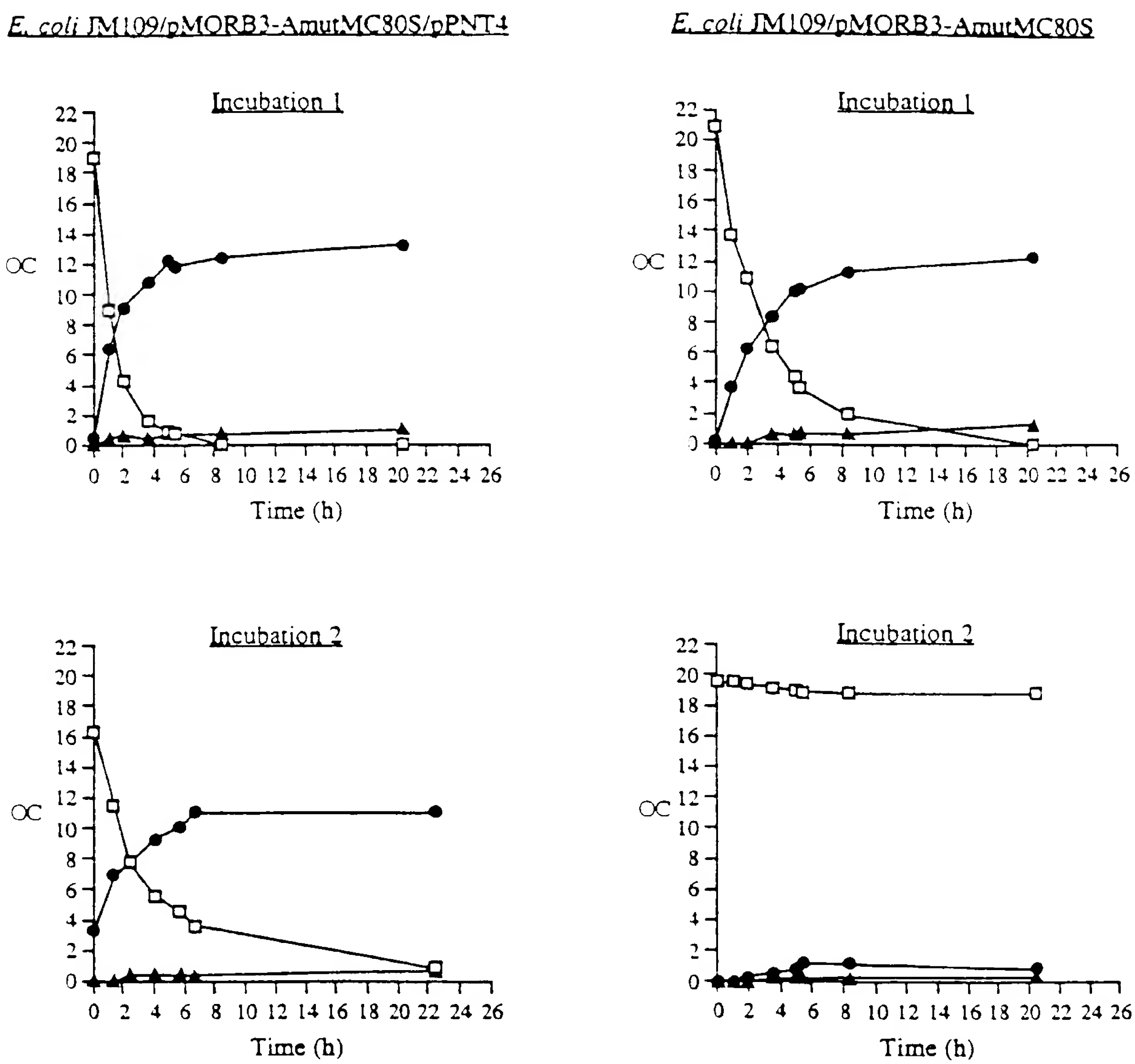
E. coli JM109/pMORB3-AmutMC80S/pPNT4
E. coli JM109/pMORB3-AmutMC80S
Incubation 1
Incubation 2
OC
Time (h)

FIGURE 4

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/02983

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N9/02 //C12P17/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	EP 0 733 712 A (AJINOMOTO CO., INC.) 25 September 1996 see page 3, line 1 - page 6, line 9 ---	1-6,8
Y	WERMUTH B ET AL: "PYRIDINE NUCLEOTIDE TRANSHYDROGENASE FROM PSEUDOMONAS AERUGINOSA: PURIFICATION BY AFFINITY CHROMATOGRAPHY AND PHYSICOCHEMICAL PROPERTIES" ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, vol. 176, no. 1, 1 January 1976, NEW YORK US, pages 136-143, XP000571345 see the whole document --- -/--	1-6,8

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

1

Date of the actual completion of the international search	Date of mailing of the international search report
17 February 1998	11.03.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	De Kok, A

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	CLARKE D M ET AL: "NUCLEOTIDE SEQUENCE OF THE PNT A AND PNT B GENES ENCODING THE PYRIDINE NUCLEOTIDE TRANSHYDROGENASE OF ESCHERICHIA COLI" EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 158, no. 3, 1 August 1986, BERLIN DE, pages 647-653, XP000571085 see the whole document ---	1,2,4
A	EP 0 388 267 A (SOCIETE NATIONALE ELF AQUITAINE) 19 September 1990 see page 2, line 2 - page 3, line 50 ---	5,6
A	WO 90 13634 A (NATIONAL RESEARCH DEVELOPMENT CORP.) 15 November 1990 see page 1 - page 6 ---	5-7
A	BRUCE N C ET AL.: "Towards engineering pathways for the synthesis of analgesics and antitussives" ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, vol. 721, 2 May 1994, NEW YORK US, pages 85-99, XP002055944 see the whole document ---	7
P,X	FRENCH C E ET AL.: "Cloning, sequencing, and properties of the soluble pyridine nucleotide transhydrogenase of Pseudomonas fluorescens" JOURNAL OF BACTERIOLOGY, vol. 179, no. 8, August 1997, WASHINGTON US, pages 2761-2765, XP002055945 see the whole document -----	1,2,4

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/02983

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0733712 A	25-09-96	AU 8002694 A	22-05-95
		BR 9407907 A	26-11-96
		PL 314090 A	19-08-96
		SK 53796 A	01-10-96
		CA 2175042 A	04-05-95
		CN 1139956 A	08-01-97
		CZ 9601213 A	11-09-96
		HU 74840 A	28-02-97
		WO 9511985 A	04-05-95
EP 0388267 A	19-09-90	FR 2644179 A	14-09-90
		AT 107961 T	15-07-94
		JP 3202483 A	04-09-91
		US 4971668 A	20-11-90
WO 9013634 A	15-11-90	AU 643687 B	25-11-93
		AU 5647890 A	29-11-90
		CA 2055442 A	13-11-90
		DE 69021564 D	14-09-95
		DE 69021564 T	18-01-96
		EP 0471740 A	26-02-92
		GB 2231332 A,B	14-11-90
		JP 5504252 T	08-07-93
		US 5387515 A	07-02-95
		US 5298414 A	29-03-94